Volume 282, number 1, 61–64 FEBS 09618 April 1991

ADONIS 001457939100326B

Is the mitochondrial Ca^{2+} uniporter a voltage-modulated transport pathway?

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Received 4 February 1991

The influence of membrane potential ($\Delta\Psi$) on Ca^{2+} transport through the Ca^{2+} uniporter (UP) was investigated in fura-2-loaded rat heart mitochondria at physiologically relevant-submicromolar-external $[Ca^{2+}]$. In the absence of $\Delta\Psi$ the UP could not mediate Ca^{2+} uptake even when an 8-fold external (~500 nM) to internal (~60 nM) $[Ca^{2+}]$ gradient was present and charge compensation was provided by acetate and the protonophore, CCCP. A small (~−120 mV) and transient $\Delta\Psi$ (generated by valinomycin) resulted in a rise in matrix $[Ca^{2+}]$ only when external $[Ca^{2+}]$ exceeded 150 nM. At physiologically high (~−180 mV) and stable $\Delta\Psi$ this threshold value for Ca^{2+} uptake dropped to 15 nM. The results indicate that (1) at physiological $[Ca^{2+}]_o$, $\Delta\Psi$ in addition to being a component of $\Delta\mu_{Ca^{2+}}$ seems to be necessary for providing a transport-competent conformation for the UP; and (2) below a threshold $[Ca^{2+}]_o$ the UP cannot operate even in the presence of a high electric driving force.

Matrix $[Ca^{2+}]$; Uniporter; Membrane potential

1. INTRODUCTION

Extensive research during the last decade has clarified that the main physiologic role of the mitochondrial Ca^{2+}-transporting system is to relay the cytoplasmic Ca^{2+} signal to the matrix space thereby regulating the activity of intramitochondrial Ca^{2+}-sensitive dehydrogenases (see for reviews [1,2]). In contrast to the remarkable progress in understanding the role of mitochondrial Ca^{2+} transport, the mode of operation of the Ca^{2+} transporters themselves remains to be elucidated.

Ca^{2+} uptake is mediated through an electrogenic, ruthenium red inhibitable Ca^{2+} uniporter, driven by the Ca^{2+} electrochemical gradient ($\Delta\mu_{Ca^{2+}}$) [3,4]. However, the transport molecule has not been unambiguously identified and the functional characterization of the uniporter is incomplete as well; there is a great variability in its reported kinetic parameters [2,3] and only few data are available about its $\Delta\Psi$-dependence [5–7]. Furthermore the majority of these measurements were carried out at Ca^{2+} concentrations highly exceeding the cytoplasmic level. Considering the multitude of voltage-gated Ca^{2+} channels in the plasma membrane the question arises whether $\Delta\Psi$ in addition to being a component of the driving force has a direct influence on the uniporter molecule. In other words: is the mitochondrial Ca^{2+}-uptake pathway a voltage-modulated transport route? This specific problem was approached by using the recently developed method of loading isolated heart mitochondria with the Ca^{2+}-sensitive fluorescent dye, fura-2 [8–14]. This technique offers the advantage of the continuous monitoring of very small transmembrane Ca^{2+} movements, at physiologically relevant and stable extramitochondrial $[Ca^{2+}]$. The results indicate that $\Delta\Psi$ might exert a conformational effect on the uniporter.

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Abbreviations: $[Ca^{2+}]_m$, free matrix $[Ca^{2+}]$; $[Ca^{2+}]_o$, external free $[Ca^{2+}]$; $\Delta\Psi$, mitochondrial membrane potential; TPP^+, tetraphenyl phosphonium ion; CCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

2. MATERIALS AND METHODS

Rat heart mitochondria were prepared and loaded with fura-2, in a similar fashion as described in [9]. Briefly, mitochondria (50 mg protein/ml) were incubated in the presence of 10 μM fura-2/AM for 5 min at 30°C, and then washed and resuspended in the preparation medium to obtain the stock suspension.

Fluorescence measurements were carried out in 2 ml of a basic medium containing 250 mM sucrose, 20 mM Tris-HCl, 3 μM rotenone, pH 7.4, using a Perkin-Elmer 3000 spectrofluorimeter (excitation at 340 nm, emission at 500 nm). Stable free Ca^{2+} concentrations of the medium were obtained with Ca^{2+}/EGTA buffers containing 4 mM EGTA and different concentrations of $CaCl_2$ calculated using the stability constant and program published by Fabiato and Fabiato [15]. The values for free $[Ca^{2+}]$ higher than 100 nM were checked by a Ca^{2+}-selective electrode [8] and below this value by the fluorescence of fura-2 free acid. Intramitochondrial free $[Ca^{2+}]$-s were calculated using the formula $[Ca^{2+}]_m = K_d(F-F_{min})/(F_{max}-F)$. F_{max} was determined in the presence of 1 μM ionomycin plus 1 μM CCCP at a $[Ca^{2+}]_o > 50$ μM, while the fluorescence of the Ca^{2+}-free dye was calculated as $F_{min} = F_{Mn} + (F_{max}-F_{Mn})0.15$, where F_{Mn} is the fluorescence in the presence of 4 mM $MnCl_2$, i.e. when also the Ca^{2+}-independent fluorescence of fura-2 is quenched. The K_d of intramitochondrial fura-2 was assumed

to be 135 nM [8,9]. After abolishing transmembrane Ca^{2+} gradients with ionomycin plus CCCP the calculated values for $[Ca^{2+}]_m$ were in good agreement with the measured $[Ca^{2+}]_m$-s in the concentration range of 0–800 nM. Considering the variations of the published values for both the intramitochondrial K_d of fura-2 [8–14] and for the affinity constant of EGTA [16], the calculated direction of the ion gradients was always verified on the basis of ion movements induced by a specific ionophore.

Membrane potential was estimated by a TPP^+ selective electrode [17] in the basic medium supplemented with 1.5 μM TPP^+. TPP^+ uptake in the presence of 3 μM rotenone, 2 μg/ml antimycin A and 2 μg/ml oligomycin was taken as passive binding to mitochondrial membranes.

3. RESULTS

3.1. *Comparison of Ca^{2+} transport via an artificial pathway, ionomycin and the Ca^{2+} uniporter in the absence of generated membrane potential*

The initial free matrix $[Ca^{2+}]$ ($[Ca^{2+}]_m$) of fura-2-loaded heart mitochondria suspended in a Ca^{2+}-free medium (containing 1 mM EGTA) ranged between 60–100 nM in different mitochondrial preparations. In the absence of respiratory substrate, the level of $[Ca^{2+}]_m$ generally remained unchanged when mitochondria were incubated in a sucrose-based medium with a $[Ca^{2+}]$ ($[Ca^{2+}]_o$) buffered at 500 nM. (In those cases when a small 'spontaneous' uptake occurred, the increase in $[Ca^{2+}]_m$ was completely prevented by the addition of KCN and oligomycin indicating that the process was driven by the oxidation of endogenous substrates or ATP hydrolysis.) Thus, in intact mitochondria no Ca^{2+} uptake could be observed in the absence of $\Delta\Psi$ generation. This fact could reflect that without charge compensation any measurable Ca^{2+} influx is hindered due to the electrogenic nature of the Ca^{2+} uniporter. However it cannot be excluded that the role of $\Delta\Psi$ in Ca^{2+} uptake is not purely thermodynamic but it has also an influence on the Ca^{2+} transporting properties of the uniporter itself. We approached this problem by comparing Ca^{2+} permeation in the absence of $\Delta\Psi$ through an artificial electroneutral pathway, ionomycin, and through the uniporter under conditions where the overall electroneutrality of the process is provided (see below). Fig. 1A shows a typical experiment when the original $[Ca^{2+}]_m$, calculated to be 63 nM, was stable at 500 nM $[Ca^{2+}]_o$. Addition of the $Ca^{2+}/2H^+$ exchanger, ionomycin substantially increased $[Ca^{2+}]_m$ practically dissipating the Ca^{2+} concentration difference across the membrane. It was important to check that this effect of ionomycin was truly due to the initiation of a transmembrane Ca^{2+} movement driven by the inwardly directed Ca^{2+} gradient because Ca^{2+} uptake through this ionophore could also be the consequence of a ΔpH, if the matrix was more acidic than the medium. The role of a ΔpH as a driving force was excluded based on the following findings. (1) The addition of the protonophore, CCCP (1 μM) did not influence the amplitude of the ionomycin-induced

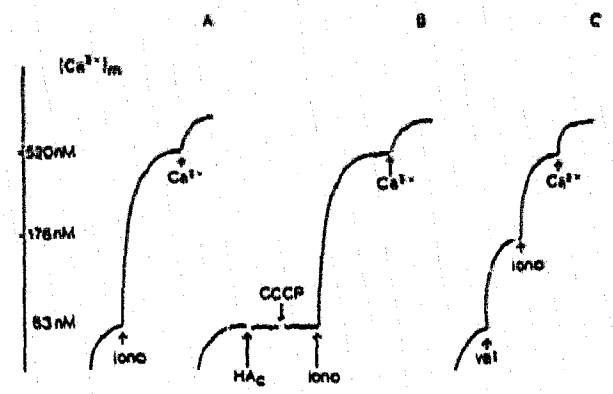

Fig. 1. Comparison of transmembrane Ca^{2+} movements mediated by ionomycin and the Ca^{2+} uniporter. Mitochondria (1 mg protein) were incubated in 2 ml of the basic medium. $[Ca^{2+}]_m$ was 500 nM. Where indicated the following additions were made: iono, 1 μM ionomycin; HAc, 5 mM Tris-acetate; CCCP, 1 μM; Val, 1 μg valinomycin; Ca^{2+}, 0.5 mM $CaCl_2$.

fluorescence signal although under these conditions both transmembrane H^+ and Ca^{2+} gradients are completely dissipated. (2) Any putative ΔpH was dissipated before, and independently of ionomycin addition by the inclusion of 20 mM KCl, 1 μM of the K^+/H^+ exchanger, nigericin and CCCP into the mitochondrial suspension [18]. This manipulation changed neither the initial fura-2 signal nor the ionomycin-induced elevation of it (not shown). We controlled that the ionomycin-mediated exchange was in fact electroneutral: no TPP^+ uptake was seen upon addition of the ionophore. Taken these findings together, the elevation of $[Ca^{2+}]_m$ in the presence of ionomycin was unambiguously due to the electroneutral Ca^{2+} influx driven by the $[Ca^{2+}]$ difference between the medium and the matrix.

The fact that respiration-inhibited mitochondria are able to take up Ca^{2+} through the uniporter if the electroneutrality of the process is maintained by the addition of the freely permeating acetic acid plus a protonophore was shown in the pioneering work of Selwyn et al. [19]. Under these conditions the overall reaction is a continuous $Ca^{2+}/2H^+$ exchange accompanied by the accumulation of Ca^{2+}-acetate. As in this classic experiment the $[Ca^{2+}]$ of the medium was very high (83 mM), the question whether the uniporter can mediate Ca^{2+} uptake into de-energized mitochondria also at physiologically relevant (submicromolar) $[Ca^{2+}]_o$ remained open. Fig. 1B shows that addition of 5 mM Tris-acetate and 1 μM CCCP did not alter $[Ca^{2+}]_m$ while ionomycin immediately exerted its $[Ca^{2+}]_m$-rising effect. Similar results were obtained with propionate, the most permeant weak acid (not shown). Thus, at physiological $[Ca^{2+}]_o$, in the absence of $\Delta\Psi$, Ca^{2+} cannot enter through the uniporter, though a more than 8-fold Ca^{2+} gradient exists and the possibility of electroneutral permeation is provided.

McCormack et al. reported that at physiological $[Ca^{2+}]_o$, Ca^{2+} uptake occurred in uncoupler-treated, fura-2-loaded mitochondria only if some respiratory substrate (300 μM oxoglutarate) was present in the incubation medium [13]. The substrate demand for initiating uptake probably indicates that under these conditions some $\Delta\Psi$ must have been built up. The ongoing substrate level phosphorylation provides ATP for the F_oF_1 ATP-ase and this together with the H^+-pumps of the respiratory chain might keep a low but non-zero $\Delta\Psi$ even in the presence of protonophore. These observations indicate that $\Delta\Psi$, in addition to being a component of the $\Delta\mu_{Ca^{2+}}$, seems to be necessary for providing a transport-competent conformation of the uniporter.

3.2. *The operation of the Ca^{2+} uniporter in the presence of membrane potential*

In further experiments we investigated the Ca^{2+}-transporting properties of the uniporter in the presence of $\Delta\Psi$ built up by the addition of valinomycin. This way of generating an internal negative diffusion potential is convenient because it does not alter the internal pH and relatively small and transient potential changes can be obtained which are sensitive to the medium $[K^+]$. Fig. 1C shows that addition of valinomycin to non-respiring mitochondria suspended in a K^+-free medium buffered at 500 nM $[Ca^{2+}]_o$ caused an immediate increase in $[Ca^{2+}]_m$. It can be noted that even the elevated $[Ca^{2+}]_m$ remained below the $[Ca^{2+}]_o$ (see Figs 1C and 2,IIA) while the subsequently added ionomycin immediately equilibrated the concentrations. Fig. 2 summarizes the observations proving that the valinomycin-induced Ca^{2+} uptake was due to the generated $\Delta\Psi$ and was mediated by the uniporter. As shown by the TPP^+ electrode trace (Fig. 2,IA), valinomycin generated a diffusion potential (the peak value of which can be estimated −120 mV) and a simultaneous increase in $[Ca^{2+}]_m$ (Fig. 2,IIA). The elevated level of $[Ca^{2+}]_m$ was maintained after the decay of $\Delta\Psi$. Addition of 2 μM Ruthenium red did not influence the $\Delta\Psi$-response (Fig. 2,IB) whereas it completely prevented the rise in $[Ca^{2+}]_m$ (Fig. 2,IIB). Inclusion of 20 mM KCl into the medium abolished both the valinomycin-induced potential change (Fig. 2,IC) and the increase in $[Ca^{2+}]_m$ (Fig. 2,IIC). The participation of the Na^+/Ca^{2+} exchanger in Ca^{2+} uptake was excluded since its specific inhibitor, diltiazem did not alter the observed phenomena.

In the following experiments we investigated the dependence of the valinomycin-induced change in $[Ca^{2+}]_m$ on $[Ca^{2+}]_o$ (Fig. 3). In the absence of any ionophore, $[Ca^{2+}]_m$ remained at basal level in the $[Ca^{2+}]_o$ range of 100 and 1100 nM. Ionomycin increased $[Ca^{2+}]_m$ in the whole concentration range indicating that even the lowest $[Ca^{2+}]_o$ was higher than $[Ca^{2+}]_m$. (CCCP did not alter the signal.) Addition of valinomycin raised $[Ca^{2+}]_m$ only when $[Ca^{2+}]_o$ exceeded 150 nM. It should be noted that at each $[Ca^{2+}]_o$ tested both components of $\Delta\mu_{Ca^{2+}}$ (i.e. $[Ca^{2+}]$ gradient and $\Delta\Psi$) favoured Ca^{2+} uptake. In spite of this fact below a threshold $[Ca^{2+}]_o$ (between 150 and 200 nM in five separate experiments under these conditions) $[Ca^{2+}]_m$ did not rise. A plausible interpretation of this finding is that the presence of $\Delta\Psi$ is a necessary but not sufficient condition. The other prerequisite is a given $[Ca^{2+}]_o$, the level of which might be dependent on the magnitude of $\Delta\Psi$ (see below). However it cannot be excluded at the moment that even the fura-2 method

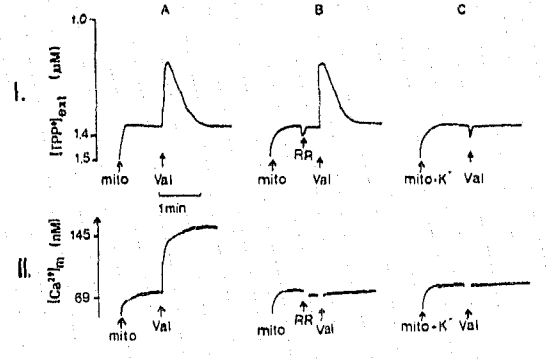

Fig. 2. Uniporter-mediated increase in $[Ca^{2+}]_m$ of fura-2-loaded heart mitochondria induced by a transient diffusion potential. Mitochondria (2.8 mg and 1.4 mg protein for I and II, respectively) were incubated in the basic medium at a $[Ca^{2+}]_o$ of 500 nM. Where indicated the following additions were made: val, 1 μg valinomycin; RR, 2 μM Ruthenium red; K^+, 20 mM KCl. Initial decrease in the $[TPP^+]$ was identical in the presence of oligomycin plus antimycin (see section 2) and was taken as passive attachment.

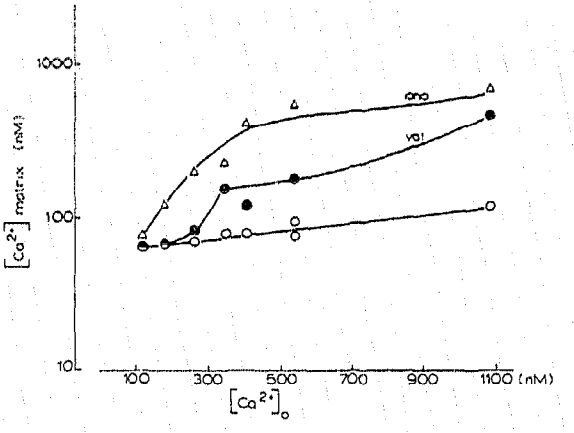

Fig. 3. Dependence of the valinomycin and ionomycin induced Ca^{2+} uptake on $[Ca^{2+}]_o$. Mitochondria (1 mg protein) were incubated in 2 ml of the basic medium at the indicated values of free $[Ca^{2+}]_o$. Fluorescence was measured in the absence of any ionophore (○). Then 1 μg valinomycin was added and the change in fluorescence was recorded in the same incubation (●). Thereafter 1 μM ionomycin was added (△). Identical Ca^{2+} levels were obtained when ionomycin was added in the absence of valinomycin. The figure shows one representative experiment of five similar ones.

(which otherwise is the most sensitive one at present) fails to detect the very small Ca^{2+} uptake that might occur during the short duration of this small $\Delta\Psi$.

Finally we tested whether a threshold $[Ca^{2+}]_o$ exists also at physiological and long-lasting $\Delta\Psi$. A greater than −180 mV $\Delta\Psi$ was generated by succinate. In the case of these respiring mitochondria the lowest $[Ca^{2+}]_o$ necessary to cause an observable increase in $[Ca^{2+}]_m$ dropped significantly, nevertheless at and below 15 nM, $[Ca^{2+}]_m$ remained on the basic level in spite of the enormous electrical driving force. The existence of this threshold is in good agreement with previous observations showing that EGTA, probably by removing Ca^{2+} from a proposed regulatory site on the uniporter, inhibits Ca^{2+} release through this transport route [20,21] while Ca^{2+} can allosterically activate its own uptake [22].

4. DISCUSSION

We propose a dual role for $\Delta\Psi$ in mitochondrial Ca^{2+} uptake. The membrane potential, beside representing the major driving force, also exerts an effect on the uniporter molecule that seems to be indispensable for the flux of Ca^{2+} across it, at least when $[Ca^{2+}]_o$ is in the (sub)micromolar range. In this respect the uniporter can be regarded as a voltage-modulated transport pathway. Though this concept has never been declared explicitly, some reports dealing with the $\Delta\Psi$-dependence of Ca^{2+} uptake contain data that are in agreement with this conclusion. Åkerman [5] analysed the initial rate of Ca^{2+} uptake (at 10 μM) as a function of $\Delta\Psi$, varied by different concentrations of 2-heptyl-4-hydroxyquinoline *N*-oxide. He found a linear current/voltage relationship, however the line did not reach the origo but intersected the abscissa at around −50 mV. (He interpreted this finding as a consequence of an 'energy-independent' Donnan potential that could not drive Ca^{2+} uptake.) Wingrave et al. [7] measured the uptake rate/$\Delta\Psi$ relationship at near-micromolar $[Ca^{2+}]$-s and found again that the extrapolation of the line intersected the $\Delta\Psi$ axis. It should be kept in mind that the $\Delta\Psi$ values at '0 Ca^{2+} uptake' were obtained by extrapolation, as the maintenance and accurate measurement of stable $\Delta\Psi$ below 100 mV is cumbersome. The cited papers thus have indicated the absence of Ca^{2+} uptake if $\Delta\Psi$ was below a given value. Our results complete this view by showing that in the absence of $\Delta\Psi$, Ca^{2+} cannot penetrate through the uniporter although the process would not be thermodynamically restricted. There is a further, though indirect indication that the uniporter might be a voltage-dependent pathway. Bernardi et al. reported an electrogenic Na^+ uniport that can be induced by removal of external Mg^{2+} [23]. This transport route was found to be voltage-gated, being quiescent below a $\Delta\Psi$ of −120 mV. On the basis of the inhibitory properties of Ruthenium red and La^{3+} we suggested that the Mg^{2+}-regulated Na^+ channel and the Ca^{2+} uniporter could be identical [24]. Finally, based on the newly introduced mitochondrial patch-clamp technique it was proposed that the inner membrane contained different voltage-dependent ion channels [25–27]. We suggest that one of them might be the Ca^{2+} uniporter.

Acknowledgements: We are indebted to Dr G.L. Lukács for valuable discussions and to Mrs E. Seres-Horváth for excellent technical assistance. Experimental work was supported by Grant OTKA/190 and by a grant from the Hungarian Ministry of Health.

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